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THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE DEVELOPMENT OF NORMALLY FERTILIZED *ARBACIA* AND *ASTERIAS* EGGS.

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The belief that cessation and initiation of development in the marine egg depended in some manner on the ionic equilibria of sea water led Loeb (1) to examine the influence of changes in the concentration of H- and OH-ions on the development of normally fertilized eggs. He found that the development of the eggs of *Arbacia* is retarded and finally prevented if increasing quantities of acid are added to sea water, and that the development to the pluteus stage is accelerated in alkaline sea water. The latter fact was indicated by the advanced size and development of the plutei formed from the treated eggs as compared with controls. On subsequent investigation he concluded that alkali does not accelerate the early cleavage rate, but only the later development from the blastula to the pluteus. The addition of excessive quantities of alkali had an injurious effect. The maximum stimulation was observed when 1.75 cc. *N*/10 NaOH were added to 100 cc. sea water. He attempted to raise the newly fertilized eggs of *Strongylocentrotus* in a neutral Ringer's solution without success, but found that with the addition of a small quantity of KOH, or better $NaHCO_3$, good larvæ might be obtained. He concluded that a neutral or faintly alkaline solution is necessary for normal development (2). This conclusion was reached from other points of view by Herbst (3) and Peter (4).

Moore, Roaf and Whitley (5) performed similar experiments with the eggs of *Echinus esculentus;* the addition of small amounts

of alkalies or alkaline salts, such as Na_2HPO_4, to sea water in which the eggs were growing caused an increase in the rate of growth in the early as well as the late stages, but larger amounts led to abnormal division. They pointed out that in some eggs in quite alkaline solutions nuclear division occurred without cytoplasmic division, so that the blastomeres became multi-nucleated. Still larger amounts of alkali inhibited both nuclear and cytoplasmic division. On the other hand, the smallest amount of acid had only an inhibitive action. There was no tendency for nuclear division without cytoplasmic division; and with comparatively small amounts of acid cell division was completely prevented. They concluded that the extreme limits of reaction at which cell division is possible lie very close together, and they pointed out that the phosphates and carbonates in sea water have a "steadying action" against fluctuations in the concentrations of H- and OH-ions which must be advantageous to cell growth. Subsequently, Whitley (6) found that small quantities of acid and alkali were very injurious to the developing eggs of the plaice, *Pleuronectes platessa*. No accelerating effect was observed in alkaline sea water, but Whitley concluded that a disturbance of the equilibrium towards the acid side is much more fatal than the opposite. There appeared to be an increase in resistance to unfavorable reactions developed in proportion to the age of the larvæ.

Glaser (7) repeated Loeb's experiments with *Arbacia* in another connection and concluded that accelerated development in alkaline sea water is limited to the development from the blastula to the pluteus, and that the early rate of cleavage is not accelerated, and may even be suppressed. Glaser noted the time required for the successive cleavage planes to appear in the majority of eggs in the cultures. By this method a small change in velocity of development would be difficult to detect, though it would become manifest if continued to the later stages where its results would of course be magnified.

Although it had another objective, the excellent work of Medes (8) on the causes of variation in the larvæ of *Arbacia* is of interest in this connection. Medes made careful comparative measurements on the skeletons of plutei obtained by inseminating and rais-

ing the eggs in sea water to which various substances had been added. She found that HCl, CO_2 and acetic acid markedly retarded development. In view of Loeb's statement that alkali does not have any effect on the early development of *Arbacia,* she re-examined this point by counting the number of divided eggs one hour after insemination, and by comparing the skeletal development of the larvæ 18 hours after insemination. She found a definite acceleration from one to 18 hours with NaOH (greatest in 1.33 cc. *N*/10 NaOH + 98.66 cc. sea water) and with Na_2CO_3 (greatest in 0.4 cc. 0.45 *M.* Na_2CO_3 + 99.6 cc. sea water), though in later stages the alkali cultures showed a retardation so that ultimately they lagged behind the controls. Larger quantities of NaOH and Na_2CO_3 inhibited development from the beginning. NaCl produced slender, perforated skeletons with conspicuous processes; there was inhibition during early development and excessive growth during later periods. NaOH led to irregularity and asymmetry, while $NaHCO_3$ increased the bulk of the skeleton with a strong tendency for regularity and symmetry.

Richards (9) has recently observed acceleration of the early cleavage rate of the eggs of the opisthobranch, *Haminea virescens.* in sea water to which NaOH and KOH had been added. No acceleration was observed after the addition of $Ba(OH)_2$ and $Cr(OH)_3$.

In none of the investigations cited were the H-ion concentrations determined or controlled, nor was allowance made for any specific influence which the carbonic acid present in acidified sea water might have. Knowing that carbonic acid inhibits cell division at H-ion concentrations which otherwise are innocuous (10), it is important to determine the limits of reaction of CO_2-free sea water within which normal development is possible.

In performing the experiments reported in this paper, *Arbacia* and *Asterias* eggs were inseminated in sea water and subsequently transferred to 100 cc. of the pH solutions prepared as described in a previous paper (11). At appropriate intervals samples of 3 to 5 cc. were removed from each lot and fixed by the addition, in the case of Arbacia, of 2 or 3 cc. of a 1–1000 solution of formalin in sea water; the *Asterias* eggs were fixed by adding 2 or 3 cc. of a 1–1000 mercury bichloride solution in sea water. These meth-

ods of fixation stop all developmental processes at once and the cleavage planes remain clearly visible for many hours. Careful counts were then made on each sample, noting the number of eggs which were undivided and the number which were in each stage of division. By multiplying the number of two-cell eggs by one, the 4-cell eggs by two, the 8-cell eggs by 3, etc., and dividing the total number of divisions by the total number of eggs, the number of divisions per egg in each sample was determined. This figure is an arithmetic index of the degree of development, or if expressed in terms of time, of the velocity of development. By counting two to 3 hundred eggs in each sample, considerable accuracy can be obtained.

A particular culture of eggs will develop under constant conditions in sea water with a mean velocity that remains practically constant so long as the number of cleavages can be accurately counted. Certain individuals will be slower than the mean and others will be faster than the mean, expressing differences in viability or developmental capacity. Such differences may be interpreted from a statistical point of view to indicate the fluctuations which any individual may undergo, and the mean to represent the behavior of the average individual. The variations observed in the development of different cultures present many interesting features which we cannot discuss at this time. It should be pointed out, however, that for studies of developmental velocity under normal and abnormal conditions, the ideal condition is to have a maximum distribution of variants ("slow" eggs and "fast" eggs) so that development will progress over short intervals of time (*i.e.*, 15 to 20 minutes) in a uniform manner. Though this condition usually obtains, there are times in the season when the eggs are in such uniform physiological condition that they divide almost simultaneously. At such times the number of divisions per egg increases by abrupt steps. This circumstance can be alleviated by averaging two successive observations on each culture. For the present purposes it will suffice to consider the mean development during the entire period of observation.

The influence of reaction on the early developmental rate of normally fertilized *Asterias* and *Arbacia* eggs is shown in Fig. 1. The data summarized in this figure are taken from several experi-

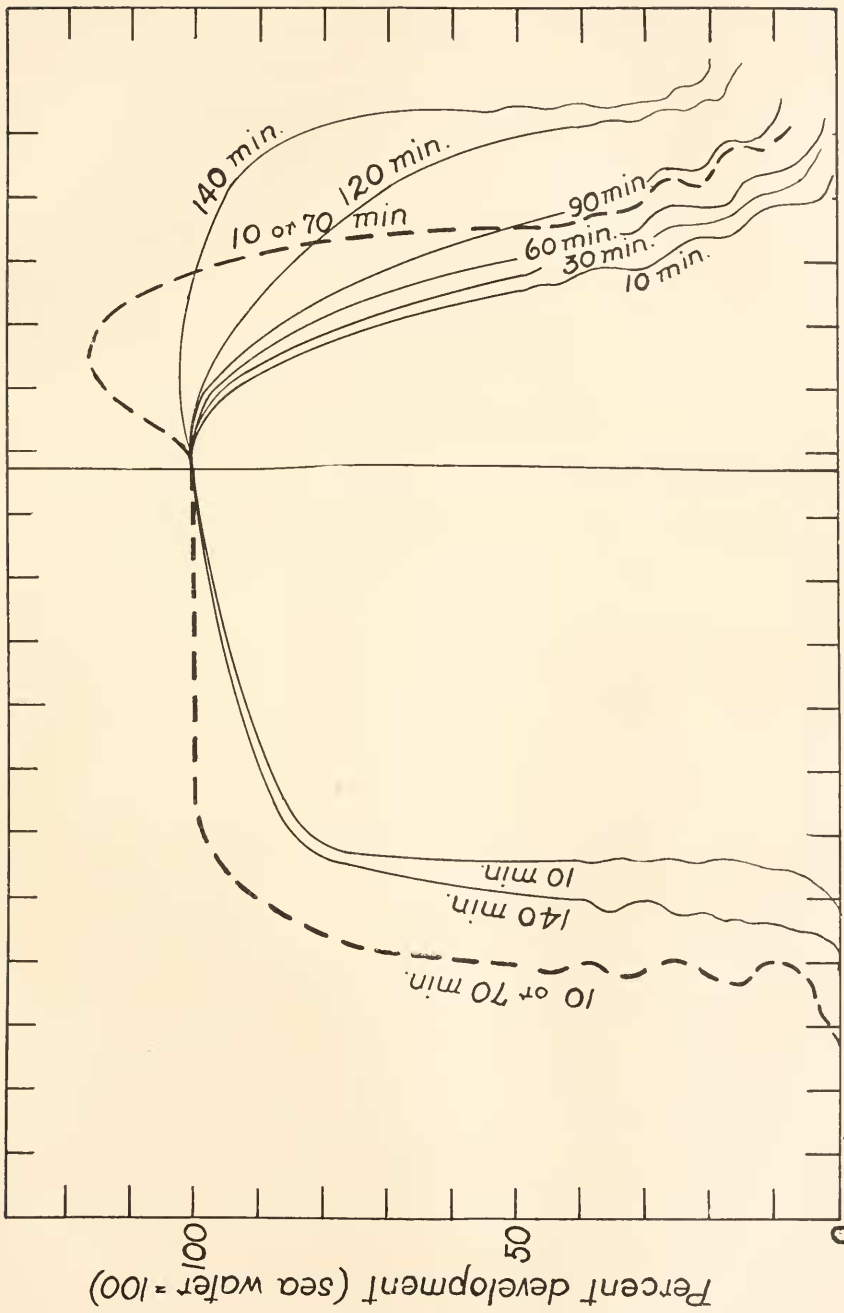

FIG. 1. The influence of H-ion concentration on the early development of normally fertilized *Arbacia* (dotted) and *Asterias* (solid line) eggs. The curves are based on the average results obtained from several experiments with each species and are expressed on the basis of the development in sea water (pH 8.15) as 100 per cent. The figures by each curve show the time after insemination at which the eggs were transferred from sea water to the acid and alkaline solutions.

ments with each species performed during the summers of 1922 and 1923. In these experiments the eggs were inseminated in sea water, centrifuged at various intervals after insemination and transferred to the pH solutions. The data given beside the curves show the time after insemination at which the eggs were transferred from sea water to the pH solutions. The development was followed quantitatively on samples taken every 20 minutes in which the number of divisions per egg was determined by careful counts. The mean development was then obtained by averaging all the observations for each solution, and the results expressed in terms of the corresponding figure for sea water as 100 per cent. The full ordinate indicates the H-ion concentration of sea water.

The dotted line in Fig. 1 refers to *Arbacia* and the solid lines to *Asterias*. The significance of the wavy portions of these lines will be discussed later. In *Arbacia* the velocity of division remains practically constant from pH 8.15 (the H-ion concentration of sea water) to pH 6.0; at 5.0 velocity of division is reduced by one half and at 4.6 division is completely suppressed. A slight increase in the alkalinity of sea water increases the velocity of division; this stimulation reaches its maximum about pH 8.8, and amounts to a 15 to 25 per cent. increase over the velocity in sea water. Above pH 8.8 there is an abrupt retardation so that the developmental velocity is reduced by one half at 9.6, and at 10.12 only a small fraction of the eggs divide even once.

Attention is called to the fact that the limiting reactions are characterized, not by a gradual, but by an abrupt inhibition of cell division within a comparatively narrow range. Between these limiting reactions cell division is essentially unimpaired.

It will be convenient for purposes of reference to define the critical limit as the pH at which the curve under consideration is reduced to its midpoint, *i.e.*, to 50 per cent. Accordingly the limits for *Arbacia* may be said to be pH 5.0 and 9.6. These limits are the same whether the eggs are placed in the pH solutions 10 minutes or 60 minutes after insemination. In the latter case, however, the degree of stimulation by alkali is slightly less.

The *Asterias* egg differs from the *Arbacia* egg notably in this—that while the resistance of the latter to both acid and alkali appears to be the same 10 minutes and 60 minutes after fertilization,

the resistance of the *Asterias* egg is much lower 10 minutes after fertilization than it is later on. There is a gradual increase in resistance, particularly in alkali, from 10 minutes after fertilization until the first cleavage, at which time the maximum resistance appears to be reached. Reference to Figure 1 will show that development is inhibited at pH 9.2 if the eggs are transferred to this solution 10 minutes after fertilization; but if the eggs are not transferred until the majority have reached the two cell stage (*i.e.*, about 140 minutes) they not only tolerate pH 9.2 but they tolerate equally well pH 10.0. This difference in resistance is strikingly shown by the cultures 24 hours later. When placed, for example, in pH 9.2 10 minutes after fertilization the velocity of development is greatly reduced; in many eggs the nucleus divides without cytoplasmic division; fragmentation and abortive division are predominant and no egg progresses beyond the 32-cell stage. When placed in this same solution after the first cleavage has occurred 90 per cent. of the eggs will develop to practically normal swimmers. The increase in resistance to acid is considerably less, the limiting acidity shortly after fertilization being pH 5.6, and 140 minutes after fertilization pH 5.4. The increase in resistance to alkali which gradually appears as the egg approaches the first cleavage plane is not to be confused with the period of great susceptibility which follows the event of fertilization, or with the periodic changes in resistance to various destructive agents which a number of observers have shown to be associated with the process of cleavage. The shortest interval after fertilization at which we transferred the eggs to the pH solutions was 10 minutes, and therefore the period of great susceptibility to destructive agents immediately following fertilization was avoided. And since in our experiments the eggs are left in the pH solutions until the conclusion of the experiments, which cover in the case of *Asterias* 5 cleavages, any periodic fluctuations in resistance, if they occur, are translated into a mean.

There is little increase in the velocity of division in alkaline solution, 2 or 3 per cent. being the maximum observed in any of our experiments. There is frequently a marked stimulation shortly after transferring to the alkaline solution, but this is transient and is followed by a decrease in the velocity of division, so

that after 4 or 5 cleavages the alkali cultures are about even with the controls. There is a slight but perceptible decrease of the velocity of division in acid solutions between pH 5.8 and 7.8, in contrast to the *Arbacia* egg where the velocity remains practically constant.

If we consider the limits for eggs transferred to the pH solutions at the time of the first cleavage, these limits are pH 5.4 and 10.1. Thus the limits within which the development of *Asterias* eggs is possible are distinctly on the alkaline side of those for *Arbacia* eggs.

In those solutions in which the velocity of development is reduced below 50 per cent., the quality of cell division in both *Arbacia* and *Asterias* eggs is greatly altered. The division of the cytoplasm is apparently restrained before the division of the nucleus, and in consequence the majority of eggs become multinuclear. This condition of abnormal division is indicated in Fig. 1 by the wavy portions of the curves. After two or 3 cleavages of the nucleus without cytoplasmic division the egg usually divides abruptly into more than two blastomeres, but the division is invariably abnormal and either soon ceases entirely or leads to cytolysis. In some cases it can be observed that the cytoplasm begins to divide but the furrow melts and the blastomeres fuse. The tendency for nuclear division without cytoplasmic division is much more marked in alkaline than in acid solutions. A point is reached on the alkaline side, however, where nuclear as well as cytoplasmic division is completely inhibited. A similar repression of cytoplasmic division without complete repression of nuclear division has been observed with lack of oxygen, the action of chloroform and ether, the action of hypertonic and hypotonic sea water, cold and other agents (12).

We are concerned here principally with variations in developmental velocity which are made manifest in the early history of the dividing egg, during that period of time in which accurate quantitative information can be obtained. It is of interest to consider, however, the effects of longer exposures. A method is not available for expressing these effects quantitatively but a fair idea of the degree of retardation during a 24 hour exposure can be obtained by comparing the general development of the larvæ.

Such comparisons have shown that some retardation of development occurs even at pH 7.6 and 8.5, and that the effect of increasing acidity or alkalinity does not take the form of an abrupt inhibition at any point, but manifests itself in almost imperceptible gradations from normal development to no development at all. In the acid solutions the inhibition culminates in coagulation with little division; and in alkaline solutions in either complete cytolysis or in the formation of formless, ciliated masses of protoplasm swimming within the fertilization membrane. It is doubtful if normal development can be obtained throughout a period of 24 hours in solutions more acid than pH 7.8, or more alkaline than 8.4.

Summary.

The effect of acid and alkaline sea water on the rate of cell division in normally fertilized *Arbacia* and *Asterias* eggs was observed as far as the 128-cell stage.

In *Arbacia,* the velocity of division is reduced to 50 per cent. of the velocity in sea water (pH 8.15) at pH 5.2 and 9.4. Between pH 5.8 and 8.2 these eggs divide normally both in respect to velocity and quality of cell division. Between pH 8.2 and 9.2 the velocity of division is increased from 15 to 25 per cent.

Asterias eggs are more sensitive to both acid and alkaline sea water during the precleavage period than at any subsequent time. When these eggs are transferred to the acid and alkaline sea water immediately after fertilization, the velocity of division is reduced to 50 per cent. at pH 5.6 and 9.2; when transferred in the two cell stage the corresponding limits are pH 5.4 and 10.2. There is a slight decrease in the mean velocity of division between pH 8.2 and 5.8, but no significant increase in solutions more alkaline than sea water.

In both species, when the developmental velocity is reduced below 50 per cent. by either acid or alkali, the nucleus tends to divide without division of the cytoplasm, and abnormal multinuclear cells are formed.

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